Essential arginine residue of the F_o-a subunit in F_oF_1-ATP synthase has a role to prevent the proton shortcut without c-ring rotation in the F_o proton channel

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INTRODUCTION

F_oF_1-ATP synthase, often simply called F_oF_1, is composed of two portions, a water-soluble F_1, which has catalytic sites for ATP synthesis/hydrolysis, and a membrane-integrated F_o, which mediates H+ (proton) translocation [1,2]. The F_oF_1 from thermophilic Bacillus PS3, which was used in the present study, as well as that from Escherichia coli, have the simplest subunit structure; α_βγδε for F_o and αβε for F_1 [3]. Proton translocation down the gradient through F_o drives rotation of a central rotor shaft made of an F_o-c oligomer ring (c-ring) and γε subunits, causing conformational changes in F_1 that result in ATP synthesis. Conversely, ATP hydrolysis in F_1 induces a reverse rotation of the rotor that forces F_o to pump protons in the reverse direction. The mechanism and structure of the F_o motor remain less clear than the F_1 motor. The stator portion of F_o motor, αβ, is located on the periphery of the c-ring [4,5]. F_o-c folds in a hairpin-like structure [6–8] with two transmembrane helices, and an essential proton-binding carboxy group (cD61 in E. coli and cE56 in Bacillus PS3) is located in the middle of the second helix. Without knowledge of atomic structure, F_o-c has been assumed to have five transmembrane helices [9,10] and the fourth helix has an essential arginine residue (αR169 in E. coli and αR169 in Bacillus PS3) [11,12]. A current model assumes that the arginine residue is located at the middle of two half-channels for proton passage in F_o-a and is close to the carboxy group in F_o-c [13–15]. In the present study, we showed that mutants with replacement of this Arg^169 of Bacillus PS3 F_oF_1 with a small or acidic residue, but not other residues, can mediate passive proton translocation. This translocation is dependent on an essential carboxy group in F_o-c, but not on c-ring rotation. It appears that Arg^169 plays a role to block a futile proton shortcut via cE56 and ensures proton-coupled rotation of the c-ring.

MATERIALS AND METHODS

Proteins and vesicles

F_oF_1 from thermophilic Bacillus PS3 with a tag of ten histidine residues at the N-terminus of the β-subunit was used throughout the present study. Culture of E. coli cells expressing thermophilic Bacillus PS3 F_oF_1, and purification of wt (wild-type) and mutant F_oF_1 with Ni-NTA (Ni2+-nitrilotriacetate) affinity chromatography were carried out as described previously [5]. Inverted membrane vesicles (membrane vesicles, hereafter) from E. coli cells were prepared as described in [5], except that MgCl_2 was omitted from the buffers. F_oF_1-stripped membrane vesicles were prepared by treatment with 2 mM EDTA as described previously [3]. Proteoliposomes were reconstituted using a freeze–thaw method [5] with some modifications. Liposomes (100 μl, 44 mg/ml) and F_oF_1 (20 μl, 10 mg/ml) were mixed vigorously, left at 25°C for 15 min, and frozen in liquid nitrogen. The suspension was thawed, added to 1 ml of water, and centrifuged (180 000 g, 30 min, 4°C). The precipitate was suspended in 1 ml of 0.5 M KCl, incubated at 55°C for 30 min, chilled in ice, added to 5 μl of MgSO_4 and centrifuged (180 000 g, 30 min, 4°C). The precipitate was suspended in 50 μl of 10 mM Hepes/NaOH (pH 7.5), 0.25 M sucrose and 5 mM MgSO_4, and used for experiments immediately.

Mutants of F_oF_1

Plasmids for F_oF_1 mutants were made from pTR19-ASDS [5] using the megaprimer method and were used for transformation of a F_oF_1-deficient E. coli strain DK8 [16]. F_oF_1 with a fused c ε α subunit [cεαF_oF_1], in which a cεα-polypeptide and F_o-a subunit was fused by a linker LAGLVPGRSP (underlined residues are the thrombin-recognition sequence), was obtained as follows. A
region containing an F$_{c}$-a gene in pTR19-ASDS was amplified by PCR to introduce a new NheI site at an upstream region of the F$_{c}$-a gene and new SpeI and PstI sites at a downstream region of the F$_{c}$-a gene. The DNA fragment obtained was digested with EcoRI and PstI and ligated into the pTR19-ASDS previously digested with both enzymes (pTR19-NASP). A region containing a gene for c-dimer (F$_{c}$-c$_{1}$) of the expression plasmid pTR-AL2 for F$_{c}$F$_{i}$ [3] was amplified by PCR to introduce a new EcoRI site at an upstream region of the F$_{c}$-c$_{1}$ gene and new NheI and PstI sites at a downstream region of the F$_{c}$-c$_{1}$ gene. The DNA fragment obtained was digested with EcoRI and PstI and ligated into the pTR19-ASDS previously digested with both enzymes to obtain a recombinant DNA for F$_{o}$F$_{1}$ [3] amplified by PCR to introduce a new EcoRI site at an upstream region of the F$_{o}$-c$_{1}$ gene and new NheI and PstI sites at a downstream region of the F$_{o}$-c$_{1}$ gene. The DNA fragment obtained was digested with EcoRI and PstI and ligated into the pTR19-ASDS previously digested with both enzymes to obtain a recombinant DNA for F$_{o}$-c$_{1}$ (pTR19-C10N), pTR19-C10N was digested with EcoRI and NheI and digested into the pTR19-AL8C [3] previously digested with EcoRI and AvrII to obtain a recombinant DNA for c$_{10}$-a gene (pTR19-C10NASP), pTR19-C10NASP was digested with EcoRI and SpeI and ligated into the pTR19-ASDS previously digested with both enzymes to obtain the recombinant DNA (pTR19-C10TA) for (c$_{10}$-a)$^{30000}$F$_{o}$F$_{1}$. A plasmid to express (c$_{10}$-a)$^{30000}$F$_{o}$F$_{1}$ with a replacement of Arg$_{169}^{\text{a}}$ of F$_{o}$-a with a lysine residue [(c$_{10}$-a)R169K]$_{F_{o}}$F$_{1}$, alanine residue [(c$_{10}$-a)R169A]$_{F_{o}}$F$_{1}$ or glutamate residue [(c$_{10}$-a)R169E]$_{F_{o}}$F$_{1}$ was constructed by the same method described above. Plasmids obtained as above were individually expressed in an F$_{o}$-deficient E. coli strain JJ001 [17] (a gift from Dr J. Hermolin, University of Wisconsin Medical School, Madison, WI, U.S.A.). When indicated, (c$_{10}$-a)$^{30000}$F$_{o}$F$_{1}$ (500 μg in 60 μl) was treated with thrombin (50 units) in 50 mM Tris/H$_{2}$SO$_{4}$ (pH 8.0), overnight at room temperature (25°C).

**RESULTS**

**Effects of the mutation at position aR169**

Mutants of thermophilic F$_{o}$F$_{1}$ with replacement of aR169 with glutamate, alanine, valine, isoleucine, lysine, phenylalanine, aspartate or tryptophan were expressed in the membranes of the host E. coli cells at approximately half-yield of that of wt-F$_{o}$F$_{1}$. All of the mutants, except for the aR169D mutant, contained a set of eight subunits of F$_{o}$F$_{1}$ (Figure 1A). F$_{o}$F$_{1}$ containing aR169D lacked the F$_{c}$-a subunit and was not analysed further. The purified F$_{o}$F$_{1}$s were reconstituted into liposomes and ATPase activities with or without pre-treatment by DCCD were measured (Figure 1B). For wt-F$_{o}$F$_{1}$, 85% of the ATPase activity was sensitive to DCCD inhibition, which represents proton-coupled activity (Figure 1B). ATPase activities of all aR169 mutants were low, approx. 30% of wt-F$_{o}$F$_{1}$, and DCCD-insensitive. As expected, these mutant F$_{o}$F$_{1}$s did not show ATP-driven proton-pumping activity (Figure 1C). Membrane vesicles prepared from E. coli cells expressing wt-F$_{o}$F$_{1}$ synthesized ATP by respiratory NADH oxidation at a rate of 45 nmol of ATP/min per mg of protein, but vesicles containing the mutant F$_{o}$F$_{1}$s did not (<1% of wt-F$_{o}$F$_{1}$). Thus the mutant F$_{o}$F$_{1}$s with substituted aR169 lost the proton-coupled ATP hydrolysis/synthesis activity. This implies that rotation of the rotor shaft (c-ring/γεζ) of F$_{o}$F$_{1}$ is prevented in these mutants.

**Proton permeability of the aR169 mutants**

When ADP and P$_{i}$, substrates for ATP synthesis, are omitted from the assay mixtures for ATP synthesis described above, the maximum proton gradient should be built by NADH oxidation. The vesicles containing wt-F$_{o}$F$_{1}$ and the mutant F$_{o}$F$_{1}$s with the substitutions aR169L, aR169K, aR169F or aR169W maintained the maximum proton gradient (Figure 2A). The mutants of F$_{o}$F$_{1}$ with substitutions aR169E, aR169A or aR169V, however, exhibited only weak ACMA fluorescence quenching, indicating that these three mutants mediated passive proton translocation along the gradient. In contrast with wt-F$_{o}$F$_{1}$, the F$_{i}$ sector cannot block the proton permeation of the F$_{o}$ sector in these mutant F$_{o}$F$_{1}$s. Next, to test proton permeability of F$_{o}$, F$_{i}$-stripped membrane vesicles were prepared with EDTA treatment. Without the F$_{i}$ sector, wt-F$_{o}$F$_{1}$ acted as a proton channel and the vesicles containing wt-F$_{o}$F$_{1}$ showed poor quenching of ACMA fluorescence (Figure 2B). Also, vesicles containing F$_{o}$F$_{1}$ with substitutions aR169E, aR169A or aR169V meditated passive proton translocation and showed small ACMA quenching. Since membrane vesicles containing F$_{o}$F$_{1}$ (Figure 2A) and F$_{i}$ (Figure 2B) with the double substitution aR169E/aR169V did not permeate proton, the carboxy group of eE56 is involved in this proton permeation pathway. In contrast, vesicles containing mutant F$_{o}$F$_{1}$ with substitutions aR169L, aR169K, aR169F or aR169W showed maximum ACMA quenching, indicating that transfer of protons from F$_{o}$-a to a carboxy group of F$_{i}$-c would be blocked. Proton permeability of proteoliposomes containing purified F$_{o}$F$_{1}$ was also tested. In this case, KCl (0.5 M) was loaded into the inside of proteoliposomes and the inside-negative membrane potential was induced by valinomycin. As shown in Figure 2(C), protons flowed into proteoliposomes containing the
mutant $F_0F_1$s with the substitutions $aR169E$, $aR169A$ or $aR169V$. Among the three mutants, $aR169E$ was most proton-permeable, $aR169A$ the next and $aR169V$ the least proton-permeable, as observed for the membrane vesicles. The proteoliposomes containing other mutant $F_0F_1$s including $aR169E/cE56Q$, as well as wt-$F_0F_1$, blocked proton influx. From the results in Figure 2, we conclude that the three mutant $F_0$s with substitutions $aR169E$,
aR169A or aR169V can mediate the passive proton translocation and that cE56 is involved in this translocation.

Fusion of Fo-c and decamer Fc-c

To investigate whether the rotation of the c-ring would be necessary for the mutant Fo,F1s to undergo proton translocation, a ‘rotation-impossible’ mutant was produced. We previously produced (c10-a)FoF1, in which ten copies of the Fc-c subunit in the c-ring were fused into a single polypeptide, and demonstrated that (c10-a)FoF1 was active in proton-coupled ATP hydrolysis/synthesis [3]. Starting from this (c10-a)FoF1, we generated (c10-a)FoF1 in which the c-ring is made up of c10 and Fc-c connected by a linker containing a thrombin recognition sequence (Figure 3A). (c10-a)FoF1 was expressed in membranes of E. coli cells with the expression level being approximately half of that of wt-FoF1. It was remarkable that membrane insertion of a nascent polypeptide that had 25 transmembrane helices (20 for c10 and five for Fc-c) occurred normally. (c10-a)FoF1 was purified as a stable complex and SDS/PAGE showed the appearance of a new protein band above the α subunit band and disappearance of bands of c10 (overlapped with β subunit band) and Fc-c (Figure 3B). The new band was confirmed to be the fused c10-a protein by Western blotting with anti-Fc-c and anti-Fc-a antibodies (results not shown). When (c10-a)FoF1 was treated with thrombin, the c10-a band disappeared and the bands of Fc-c and c10 appeared instead. Relative staining intensities of each subunit band were similar to those of wt-FoF1, ensuring their normal subunit stoichiometry. Because c-ring rotation relative to Fc-c was impossible, (c10-a)FoF1 was unable to catalyse ATP-driven proton translocation (Figure 3B). However, as expected, the thrombin-treated (c10-a)FoF1 catalysed ATP-driven proton-pumping. Therefore subunits of (c10-a)FoF1 were assembled into a native-like structure. These results also provided evidence for the proposed membrane topology of Fc-c, i.e. periplasmic location of its N-terminus.

Proton permeation through (c10-a)FoF1 and its variants

The (c10-a)FoF1 variants with mutation of aR169E [(c10-aR169E)FoF1] or aR169A [(c10-aR169A)FoF1] were also expressed in E. coli at the same expression level as that of (c10-a)FoF1. They were purified as stable complexes (Figure 3C). Membrane vesicles containing (c10-a)FoF1 maintained the proton gradient generated by NADH oxidation (Figure 4A). Since rotation of the c-ring is prevented, (c10-a)FoF1 can no longer work as a proton translocating. In contrast, the vesicles containing (c10-aR169A)FoF1 or (c10-aR169E)FoF1 partially lost the gradient. Parallel results were obtained for the Fc-c-removed membrane vesicles (Figure 4B). The proton permeability of proteoliposomes containing purified (c10-a)FoF1 and its variants was also tested (Figure 4C). Even though the rate was low, (c10-aR169A)FoF1 and (c10-aR169E)FoF1 in the proteoliposomes catalysed passive proton translocation, but (c10-a)FoF1 did not. In any of the above tests, (c10-aR169E)FoF1 was always more efficient in proton translocation than (c10-aR169A)FoF1. Because c-ring rotation was impossible in (c10-a)FoF1 and its variants, these results provide evidence that protons can pass through Fc-c without accompanying c-ring rotation in the case when Arg169 of Fc-c is replaced with a glutamate or alanine residue.

The arginine-switched mutant blocks proton permeation

In E. coli, the essential arginine residue in Fc-c can be transferred to the position of aQ252 and this arginine-switched (aR210Q/Q252R)FoF1 retains small proton-coupled activity [11,18,19]. E. coli aQ252 corresponds to aQ217 in Bacillus PS3, and we made a Bacillus version of the arginine-switched (aR169G/Q217R)FoF1, and the activity of the membrane vesicles containing the arginine-switched mutant was compared with that of aR169G)FoF1. As far as we tested, both mutants could not catalyse ATP-driven proton translocation (Figure 5A). The arginine-switched Fc-c, but not (aR169G)FoF1, can maintain the proton gradient established by NADH oxidation (Figure 5B). When the Fc sector was removed from membrane vesicles, wt-Fc and (aR169G)FoF1 were proton permeable, but the arginine-switched Fc was not (Figure 5C). These results showed that (aR169G)FoF1 allows passive proton translocation and, similar to

Figure 3 Rotation-impossible Fo,F1 with a fused c10-a subunit

(A) A schematic illustration of a c10-a subunit. Ten copies of Fc-c subunits are fused into a single polypeptide which is again fused with Fc-c through a linker containing a sequence susceptible to thrombin protease. (B) SDS/PAGE analysis of the purified (c10-a)FoF1 before and after treatment with thrombin protease. The c10-a subunit appeared as a diffuse band above the subunit band and the c10 band was overlapped with that of the β subunit. (C) Recovery of ATP-driven proton-pumping activity of (c10-a)FoF1 by cleavage of a linker between c10 and Fc-c by thrombin. The reaction was initiated by the addition of 1 mM ATP to the proteoliposomes containing (c10-a)FoF1, with (black) or without (grey) thrombin pre-treatment. The inside acidification was monitored with fluorescence quenching of ACMA. Details are given in the Materials and methods section.
Essential arginine residue in F$_{1}$-a acts as a proton barrier in F$_{1}$-channel

**DISCUSSION**

Our observations on the mutant F$_{1}$F$_{1}$s are well explained by the ‘arginine proton barrier’ model (Figure 6A). It has been thought that, in the native F$_{1}$F$_{1}$, a proton coming through a half-channel in F$_{1}$-a is transferred to a carboxy group of F$_{1}$-c in the c-ring, enabling this F$_{1}$-c to move into the lipid-surrounding environment.

E. coli (aR210Q/Q252R)F$_{0}$ [11,18], the transferred arginine residue at 217 can prevent this.

The c-ring makes almost one revolution carrying the protonated carboxy group near the other half-channel of F$_{1}$-a, into which the proton is transferred. Shortcut of protons, that is, from one half-channel to the other via a carboxy group of F$_{1}$-c without c-ring rotation, is prevented by the arginine residue of F$_{1}$-a that would be located between two half-channels. Molecular simulation indicates that a positive charge of arginine might contribute to this function [20,21]. If this arginine residue is replaced by a small residue (glycine, alanine or valine) or an
acidic residue (glutamate), protons could have a chance to take a shortcut pathway (Figure 6B). If this arginine residue is replaced by phenylalanine, isoleucine, lysine or tryptophan, a proton is transferred to one half-channel and the F o-carboxy group of cE56 of Fo-c is brought close to the other half-channel, to which the proton is transferred. In this scheme, the proton shortcut from one half-channel to the other via cE56 without revolution of the c-ring is prevented by aR169 (B)(aR169F)F1. If aR169 of F o-a is replaced by glutamate, proton transfer via cE56 without accompanying c-ring rotation can occur. (C) (aR169F)F1F1. If aR169 of F o-a is replaced by phenylalanine (or isoleucine, tryptophan or lysine), transfer of protons between half-channels of F o-a and a carboxy group of cE56 of F c-c is blocked.

Figure 6 Arginine proton barrier model for the role of aR169 in F o-F1

Left-hand side: proton transfer from F o-a to cE56 of the c-ring. Right-hand side: proton passage through F o1. (A) wt-F o-F1. Each proton reaches the F o-a/c-ring interface through a half-channel of F o-a and is transferred to a deprotonated carboxy group of cE56. After nearly one revolution of the c-ring, the F c-carrying this protonated cE56 is brought close to the other half-channel, to which the proton is transferred. In this scheme, the proton shut-off from one half-channel to the other via cE56 without revolution of the c-ring is prevented by aR169. (B) (aR169F)F1. If aR169 of F o-a is replaced by glutamate, proton shut-off via cE56 without accompanying c-ring rotation can occur. (C) (aR169F)F1F1. If aR169 of F o-a is replaced by phenylalanine (or isoleucine, tryptophan or lysine), transfer of protons between half-channels of F o-a and a carboxy group of cE56 of F c-c is blocked.

by tightly associated F1. In the proton-permeable mutant F o,F1, proton permeation is mediated through a shortcut without c-ring rotation and is therefore not prevented by F1. Thus the conserved arginine residue of F o-a plays dual roles: to block a futile proton shortcut as a proton barrier and to facilitate proton transfer between F o-a half-channels and the F o-c carboxy group at the F o-c-a-ring interface.

AUTHOR CONTRIBUTION

Noriyo Mitome and Sakurako Ono designed and performed experiments, analysed data and wrote the paper; Hiroki Sato performed experiments and analysed data; Toshiharu Suzuki and Nobuhito Sone designed experiments and analysed data; Masasuke Yoshida designed experiments, analysed data and wrote the paper.

ACKNOWLEDGEMENTS

We thank B. Feniouk for critical reading of the manuscript prior to submission.

FUNDING

This work has been supported in part by the Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan [grant number 18107004 (to M.Y.), and grant numbers 19042006, 19770103 (to N.M.)].

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